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Functional Analysis of the Herpes Simplex Virus UL42 Protein

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The herpes simplex virus *UL42* gene encodes a multifunctional polypeptide (UL42) that is essential for virus DNA replication. To further understand the relationship between the structure of UL42 and the role that it plays during virus replication, we analyzed an extensive set of mutant UL42 proteins for the ability to perform the three major biochemical functions ascribed to the protein: binding to DNA, stably associating with the virus DNA polymerase (Pol), and acting to increase the length of DNA chains synthesized by Pol. Selected mutants were also assayed for their ability to complement the replication of a *UL42* null virus. The results indicated that the N-terminal 340 amino acids of UL42 were sufficient for all three biochemical activities and could also support virus replication. Progressive C-terminal truncation resulted in the loss of detectable DNA-binding activity before Pol binding, while several mutations near the N terminus of the polypeptide resulted in an altered interaction with DNA but had no apparent effect on Pol binding. More dramatically, an insertion mutation at residue 160 destroyed the ability to bind Pol but had no effect on DNA binding. This altered polypeptide also failed to increase the length of DNA product synthesized by Pol, and the mutant gene could not complement the growth of a *UL42* null virus, indicating that the specific interaction between Pol and UL42 is necessary for full Pol function and for virus replication. This study confirms the validity of the Pol-UL42 interaction as a target for the design of novel therapeutic agents.

Herpes simplex virus (HSV) provides an excellent model system for the study of DNA replication in eukaryotic cells in part because it encodes many if not all of the proteins that are directly required for replication of the virus genome (6). HSV is also a ubiquitous human pathogen whose control relies primarily on antiviral drugs. The ultimate target of most of these drugs is the virus-encoded DNA polymerase (Pol), although in principle, any of the essential replication proteins could serve as an excellent target (8).

The UL42 protein (product of the *UL42* gene), another of the essential replication proteins, was originally identified as a double-stranded DNA (dsDNA)-binding protein (40). More recently, it has become apparent that the virus Pol in fact consists of a heterodimer of a catalytic subunit (product of the *UL30* gene) and UL42. As first described for HSV-2 (39, 44) and more recently for HSV-1 (11, 17, 20), the two polypeptides copurify under a variety of conditions, indicating a stable association. Although the Pol polypeptide by itself has catalytic activity (31), the processivity of the enzyme on single-stranded templates is enhanced in the presence of UL42 (20, 21). The UL42-binding site on Pol has been mapped to the carboxy (C) terminus of the protein (13, 14), but little is known of the functional domains of UL42 itself or how it increases the processivity of Pol. The fact that UL42 has an intrinsic affinity for dsDNA suggests the attractive hypothesis that it tethers Pol to the template and thereby increases processivity by discouraging dissociation of the enzyme from the primer terminus (20). UL42 is essential for viral DNA replication (23, 30), but it is not known whether this derives from its role as a Pol accessory subunit or whether it performs other functions.

In this report, we present the results of experiments designed to map the regions of UL42 necessary for binding to dsDNA and Pol by analysis of an extensive set of insertion and deletion mutants. We then tested the ability of these mutants to increase the length of DNA product synthesized by Pol on a singly primed single-stranded DNA (ssDNA) template. These results permitted a correlation between the binding activities of the mutants and their ability to support Pol-driven long-chain DNA synthesis and also allowed us to make predictions regarding the organization of the domains of the polypeptide. In addition, by correlating the results of the in vitro assays with the ability of the mutants to complement the growth of a *UL42* null mutant in transfected Vero cells, we test the hypothesis that the Pol-UL42 interaction is necessary for viral growth and is therefore a valid target for the design of antiviral drugs.

MATERIALS AND METHODS

Linker insertion mutagenesis. A plasmid-encoded copy of the KOS strain *UL42* gene (pINGUL42 [14]) was pseudo-randomly linearized by digestion with the frequently cutting restriction enzyme *Hae*III (New England Biolabs), *Sal*I (Bethesda Research Laboratories), or *Acc*II (Stratagene) in the presence of ethidium bromide (38) and then linker tailed with the 12-bp palindromic oligonucleotide TGCATCGAT GCA as previously described (13). Unit-length plasmid was gel purified and transformed into *Escherichia coli* DH5 α , following which colonies were screened for intact plasmids containing a single insertion within the UL42 coding region by standard miniprep and restriction enzyme digest. The oligonucleotide was designed to include a *Cla*I restriction enzyme site and not to encode proline or stop codons in any translational reading frame. Insertions resulting in a mutant phenotype, and those insertions which could not be unam-

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iguously located by restriction enzyme mapping, were confirmed by dideoxynucleotide sequencing.

Deletion mutagenesis of UL42. The C-terminal deletion mutants Δ C478, Δ C441, Δ C375, Δ C340, and Δ C307 (numbered according to the last UL42-specific residue present) were obtained by linearizing plasmids containing the appropriate insertion mutant with *Cla*I to provide a truncated template for in vitro transcription and subsequent translation in rabbit reticulocyte lysate. Δ C269 was similarly expressed by linearizing pING42 at a *Sal*I site at nucleotide 1049 (33). To obtain constructs capable of expressing the desired C-terminal deletions after transfection into Vero cells, plasmids pING42(I-441), -(I-341), and -(I-308) were linearized as described above and then linker tailed with the self-complementary oligonucleotide CGCTAGTCTAGACTAG, which was designed to contain stop codons in all three translational reading frames, as described previously (13). Plasmid pING42 Δ 35–280 (numbers refer to the amino acids deleted) was constructed by excising the central 750-bp *Pst*I fragment from the UL42 gene followed by religation of the remaining plasmid. Plasmids pT742 Δ N35 Δ C282 and pT742 Δ N281 (N-terminal deletions are named according to the first UL42-specific residue present) were constructed by cloning the central 750-bp *Pst*I fragment and a 3' 1,350-bp *Pst*I fragment, respectively, into the *Pst*I site of plasmid pT7-7 (43). Plasmid pT742 Δ N10 was constructed by using the *Cla*I site of the insertion mutant I-9 and an *Eco*RV site 3' to the UL42 gene in the pING42 plasmid polylinker to excise all but the very 5' end of the gene. After treatment with the Klenow fragment of *E. coli* Pol I to generate blunt-ended DNA, the resulting fragment was ligated into similarly blunt-ended *Eco*RI-cut pT7-7. Similarly, plasmids encoding Δ N132 and Δ N160 were constructed by using the end-filled *Cla*I sites of mutants I-131 and I-160, respectively, except that a *Hind*III site in the polylinker was used to cleave the 3' end of the gene. The insert for Δ N132 was ligated into pT7-7 cut with *Hind*III and *Eco*RI (end filled), while Δ N160 was constructed with pT7-7 cut with *Sma*I and *Hind*III. In the latter five constructs, an in-frame ATG is supplied by the vector. In-frame internal deletions of UL42, i.e., Δ 9–20, Δ 20–131, Δ 114–159, Δ 131–191, Δ 161–223, Δ 20–191, Δ 242–250, Δ 250–308, Δ 308–356, Δ 191–250, Δ 250–376, and Δ 191–376, were all constructed in a similar fashion. Constructs containing the appropriate linker insertion mutations (Table 1) were digested with *Cla*I and *Hind*III to divide the plasmids into a vector portion containing that part of the UL42 gene 5' to the insertion and a separate DNA fragment containing the 3' end of the gene down to the unique *Hind*III site in the plasmid polylinker. After agarose gel purification, vector and insert fragments derived from plasmids containing linker insertions in the same translational reading frame were ligated together to generate the specified gene deletion. As these constructs were derived from linker insertion mutants, each encodes a four-amino-acid insertion as well as the indicated deletion.

In vitro transcription-translation and immunoprecipitation. After linearization with the appropriate restriction enzyme, plasmids were transcribed in vitro by either bacteriophage SP6 or T7 RNA Pol, and the resulting mRNA was translated in rabbit reticulocyte lysate (Promega) as previously described (14). To assay the [³⁵S]methionine-labeled UL42 mutants thus produced for the ability to bind to the HSV Pol, the reticulocyte lysate was microcentrifuged for 10 min at room temperature, and 5- μ l aliquots of the supernatant were then incubated with either 140 ng of β -galactosidase (Sigma) or 140 ng of purified HSV Pol (generous gift of K. Weissbart of this laboratory). After an incubation of 1 h at room

TABLE 1. Locations and phenotypes of linker insertion mutations in the UL42 gene

Insertion ^a	Sequence ^b	Phenotype ^c		
		Pol binding	DNA binding	Processivity
I-9	A→VHRCT	++	++	++
I-17	A→VHRCT	++	++*	++
I-20	A→VHRCT	++	++*	++
I-113	ASMH	++	++	++
I-131	A→VHRCT	++	++	++
I-160	ASMH	—	++	—
I-191	A→VHRCT	++	++	++
I-194	A→VHRCT	++	++	++
I-224	ASMH	++	++	++
I-242	A→VHRCT	++	++	++
I-250	A→VHRCT	++	++	++
I-308	A→VHRCT	++	++	++
I-341	A→VHRCT	++	++	++
I-356	A→VHRCT	++	++	++
I-376	A→VHRCT	++	++	++
I-442	A→VHRCT	++	++	++
I-478	ASMH	++	++	++

^a Mutants are designated according to the codon number disrupted by the linker insertion.

^b The amino acids encoded by the inserted linker are given in standard single-letter code. Also indicated where appropriate are the identities of amino acids altered by out-of-reading-frame insertions.

^c Scored as follows: ++, indistinguishable from wild-type UL42; —, no detectable activity; ++*, the mutant bound DNA but displayed an altered salt elution profile.

temperature, the aliquots were diluted by the addition of 100 μ l of immunoprecipitation buffer (IP buffer; 100 mM KCl, 50 mM Tris-Cl [pH 7.6], 5 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 0.02% NaN₃) containing 0.1% Nonidet P-40 and 50 μ l of a 10% (wt/vol) suspension of protein A-Sepharose (Sigma) in the previous buffer. Finally, 2 μ l of a rabbit antiserum raised against a Pol- β -galactosidase fusion protein capable of precipitating both Pol and β -galactosidase (PP5 [47]) was added, and the mixture was incubated overnight at 4°C on a rotator. The following day, the Sepharose beads were collected by centrifugation and washed twice with 750 μ l of IP buffer containing 1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS) and once with IP buffer containing 0.1% Nonidet P-40. Bound proteins were then eluted by boiling in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer and analyzed by SDS-PAGE and autoradiography.

DNA-cellulose chromatography. Aliquots of reticulocyte lysate-expressed protein (10 μ l) were diluted into 100 μ l of TM buffer (20 mM Tris-Cl [pH 7.6], 5 mM MgCl₂) containing 50 mM NaCl, treated with 2 μ l of an RNase A/T₁ mixture (RNase-it; Stratagene) for 20 min at room temperature to remove free RNA, and then applied to a 0.5-ml-bed-volume dsDNA-cellulose (Sigma) column equilibrated in the same buffer. The column was washed twice with 0.5 ml of TM buffer plus 50 mM NaCl and then sequentially eluted with 2 column volumes each of TM buffer containing 100 mM, 250 mM, 500 mM, and 1 M NaCl. The resulting 0.5-ml fractions were then concentrated by ethanol precipitation in the presence of 40 μ g of carrier bovine serum albumin (BSA; Sigma) and analyzed by SDS-PAGE.

Pol assays. Reticulocyte lysate-expressed UL42 mutants were assayed for the ability to increase the length of the DNA products synthesized by purified HSV Pol on a singly primed M13 ssDNA template. Aliquots of lysate were diluted in 50 μ l of buffer containing 100 mM (NH₄)₂SO₄, 20

Ability to:










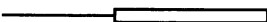















UL42 ORF		Bind Pol	Bind DNA	Stimulate Pol
	Wild Type (488 aa)	++	++	++
	ΔC478	++	++	++
	ΔC441	++	++	++
	ΔC375	++	++	++
	ΔC340	++	++	++
	ΔC307	+/-	-	-
	ΔC269	+/-	-	-
	ΔN10	++	++*	++
	ΔN132	-	-	-
	ΔN160	-	-	-
	ΔN281	-	++*	-
	ΔN35ΔC282	+/-	-	-
	Δ9-20	++	++*	++
	Δ20-131	-	-	-
	Δ114-159	-	-	-
	Δ131-191	-	-	-
	Δ161-223	-	-	-
	Δ20-191	-	-	-
	Δ242-250	++	++	++
	Δ250-308	-	-	-
	Δ308-356	-	-	-
	Δ191-250	-	-	-
	Δ250-376	-	-	-
	Δ35-280	-	-	-
	Δ191-376	-	-	-

FIG. 1. Summary of UL42 deletion mutants. Expressed portions of the UL42 ORF are represented as boxes, and deleted regions are shown as lines. Phenotypes of the mutants in three *in vitro* assays are tabulated on the right. Scores were assigned as follows; ++, indistinguishable from wild-type UL42; +/-, weak activity; -, minimal or undetectable activity; ++*, the mutant bound DNA but displayed an altered salt elution profile. Overall phenotypes are also indicated graphically by the shading of the boxes as follows: black, mutants that behaved indistinguishably from wild-type UL42 in two

mM Tris-Cl (pH 7.5), 3 mM MgCl₂, 0.1 mM EDTA, 0.5 mM dithiothreitol, 4% glycerol, 40 μg of BSA per ml, 60 μM dATP, dGTP, and dTTP, 10 μM [α-³²P]dCTP (2 μCi), 25 fmol of purified Pol (generous gift of K. Weissbart), and 50 fmol of a singly primed M13 ssDNA template prepared as described previously (13). After a 30-min incubation at 37°C, 10-μl samples were taken and analyzed by SDS-PAGE to monitor the integrity of the mutant UL42 polypeptides, while DNA synthesis was terminated in the remainder of the reaction by the addition of 50 μl of 1% SDS-10 mM EDTA-10 mM Tris-Cl (pH 8)-200 μg of proteinase K per ml. Following incubation at 37°C for 1 h, the samples were extracted once with phenol-chloroform (1:1, vol/vol) and ethanol precipitated, and the newly synthesized ssDNA was detected by alkaline gel electrophoresis (29) and autoradiography.

Transient complementation of a UL42 null mutant. Vero cells at approximately 80% confluence were transfected with 100 ng of the indicated pING42 plasmids (which contain a functional promoter) by the DEAE-dextran method. Sixteen hours later, the cells were infected with the virus CgalΔ42 at a multiplicity of infection of 3. CgalΔ42 has a 1.26-kb deletion of the UL42 gene and is therefore phenotypically null for UL42 (23; generously supplied by Paul Johnson and Deborah Parris). After virus adsorption for 1.5 h, the cells were washed twice with serum-free medium, treated for 2 min with an acid-glycine saline wash (5) to remove extracellular virus, and then washed twice with serum-free medium, after which fresh medium was added. Twenty-four hours later, virus was harvested (9) and titered on the V9 cell line, which contains a resident copy of the UL42 gene and is therefore permissive for CgalΔ42 growth (23; generously provided by Paul Johnson and Deborah Parris). Progeny were also titered on Vero cells to ensure that they did not arise from recombination events.

RESULTS

Insertion and deletion mutagenesis of UL42. To map the regions of UL42 important for its functions, we created a library of 12-bp linker insertion mutations within the UL42 gene as described in Materials and Methods. Table 1 shows the locations of the 17 mutants generated, numbered according to the amino acid residue interrupted by the insertion. As the inserted oligonucleotide contained a unique *Cla*I restriction enzyme site, plasmids containing these mutations could then be used to construct a series of N- and C-terminal deletion mutants and a set of in-frame internal deletions (Materials and Methods). Additional deletion mutants were also constructed by using convenient restriction enzyme sites within the UL42 gene. Figure 1 summarizes the sizes and locations of the 24 deletion mutants examined in this study.

Mapping the dsDNA-binding site of UL42. Since UL42 has an intrinsic affinity for dsDNA, which may be of significance for the protein's role as a processivity factor or other functions, we assayed the panel of insertion and deletion mutants described above for the ability to bind to a dsDNA-cellulose column. Aliquots of the [³⁵S]methionine-labeled polypeptides were diluted at least 10-fold in buffer containing

or more assays; grey, mutants that had some activity in one assay; and white, those that had minimal or undetectable activity in all three assays. aa, amino acids.

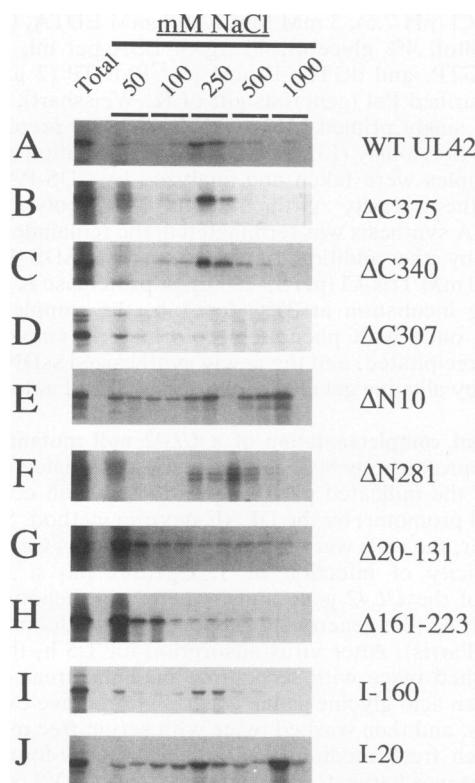


FIG. 2. DNA-binding assay. [^{35}S]methionine-labeled UL42 polypeptides expressed in rabbit reticulocyte lysate were applied to DNA-cellulose columns and eluted with buffer containing the indicated concentration of NaCl. Fractions were collected and analyzed for protein content by SDS-PAGE and autoradiography. An equivalent unfractionated amount of each sample was also analyzed (Total). WT, wild-type.

50 mM NaCl and applied to a DNA-cellulose column before stepwise elution with 2 column volumes each of buffer containing salt at the concentrations shown in Fig. 2. Fractions of 1 column volume each were collected and analyzed by gel electrophoresis after concentration by ethanol precipitation. Wild-type UL42 synthesized in rabbit reticulocyte lysate was retained by the column, with very little protein appearing in the wash fractions (50 mM). The bound polypeptide was eluted by increasing salt concentrations, with the majority of the material appearing in the 250 mM fractions (Fig. 2A). Control experiments using cellulose alone as a column matrix revealed no significant retention of the polypeptide (data not shown), indicating that the assay reflects a genuine interaction with DNA. Two C-terminal deletion mutants (ΔC375 and ΔC340) behaved like full-length UL42 in that the majority of the protein was retained by the column at 50 mM NaCl but then eluted in the presence of 250 mM NaCl (Fig. 2B and C). However, further C-terminal truncation (ΔC307 ; Fig. 2D) resulted in a polypeptide that eluted almost entirely in the first wash fraction, indicating that in 50 mM NaCl, it had very little affinity for DNA.

The smallest N-terminal truncation tested (ΔN10) displayed an altered ability to bind DNA (Fig. 2E). Like wild-type UL42, most of the polypeptide was retained by the column at 50 mM NaCl, but instead of the majority eluting at 250 mM NaCl, approximately half of the material was eluted by 1 M NaCl. ΔN10 was not retained by a cellulose column

(data not shown), indicating that the altered elution profile from dsDNA-cellulose results from an altered interaction with DNA. Mutants ΔN132 and ΔN160 eluted almost entirely in the wash fractions, indicating the loss of DNA-binding activity (Fig. 1), but unexpectedly, further truncation of the N terminus of the polypeptide apparently restored DNA binding, as mutant ΔN281 was retained by the column at 50 mM NaCl but eluted with 500 mM salt (Fig. 2F). Additionally, ΔN281 did not bind to a cellulose column (45).

We also tested 13 internal deletion mutants that removed in sum residues 9 to 376 for the ability to bind DNA. Eleven of these mutants were not retained significantly by the column at 50 mM NaCl (Fig. 2G [$\Delta\text{20-131}$] and H [$\Delta\text{161-223}$]; results for other mutants are summarized in Fig. 1). However, a small deletion near the middle of the polypeptide, $\Delta\text{242-250}$, apparently did not affect DNA binding, as the polypeptide eluted normally at 250 mM salt (Fig. 1). Interestingly, a mutant containing a small deletion near the N terminus, $\Delta\text{9-20}$, behaved similarly to ΔN10 in that it eluted in two populations, one at 250 mM and one at 1 M NaCl (Fig. 1).

The panel of 17 linker insertion mutants was also assayed for DNA-binding ability. As shown for I-160 (Fig. 2I), 15 of the insertion mutants bound DNA normally and eluted with 250 mM NaCl (summarized in Table 1). However, two insertion mutations near the N terminus (I-20 [Fig. 2J] and I-17 [Fig. 1]) eluted partially at 250 mM and partially at 1 M salt, similarly to the N-terminal deletion mutants ΔN10 and $\Delta\text{9-20}$.

The results of these experiments are summarized in Fig. 1 and Table 1; the N-terminal two-thirds of the polypeptide is sufficient for DNA binding, while the C-terminal 148 amino acids are dispensable. Consistent with this finding, all of the deletion mutants assayed that lacked more than 12 residues from within these N-terminal 340 amino acids failed to bind DNA, with the notable exception of ΔN281 , while mutations within the first 20 amino acids altered the interaction with DNA.

Mapping the Pol-binding site on UL42. Next, we attempted to map the region of UL42 responsible for binding to Pol by analyzing the ability of various deletion mutants to coprecipitate with Pol. Figure 3a shows the results obtained with wild-type and mutant UL42 polypeptides lacking 113, 148, and 219 residues from the C terminus of the protein. [^{35}S]methionine-labeled wild-type UL42 synthesized in rabbit reticulocyte lysate was immunoprecipitated by antisera directed against a Pol- β -galactosidase fusion protein when mixed with unlabeled purified Pol but not when incubated with β -galactosidase (Fig. 3aA). This specific coprecipitation is indicative of a stable complex formed between the two polypeptides. Truncated UL42 proteins lacking as many as 148 amino acids from the C terminus (ΔC375 and ΔC340) similarly coprecipitated with Pol but not with β -galactosidase (Fig. 3aB and C). Consistent with this finding, mutants containing smaller deletions of the C terminus (ΔC478 and ΔC441) also bound Pol normally (Fig. 1). However, further deletion of C-terminal sequences resulted in polypeptides that coprecipitated with Pol to a substantially reduced extent and also displayed some nonspecific precipitation with β -galactosidase (ΔC269 [Fig. 3aD] and ΔC307 [Fig. 1]).

Figure 3b shows the results of similar experiments carried out on UL42 mutants containing progressive deletions of the N terminus. The loss of nine residues from the N terminus of the polypeptide (ΔN10) did not prevent the altered UL42 protein from coprecipitating with Pol (Fig. 3bA). Larger deletions of the N terminus such as ΔN132 or ΔN281

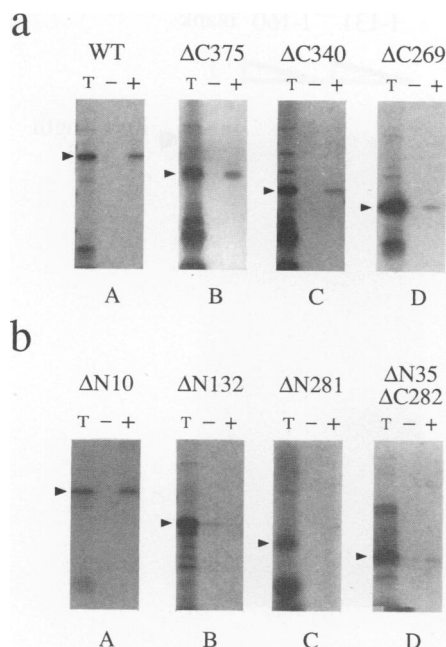


FIG. 3. Ability of N- and C-terminally truncated UL42 mutants to bind Pol. Aliquots of the indicated [35 S]methionine-labeled UL42 polypeptides expressed in rabbit reticulocyte lysate were analyzed by SDS-PAGE before (T) or after immunoprecipitation with antisera reactive against Pol and β -galactosidase in the presence of β -galactosidase (-) or Pol (+). Migration of the UL42 proteins is indicated by arrowheads. (a) C-terminal deletion mutants; (b) N-terminal and both N-terminal and C-terminal deletion mutants. WT, wild type.

destroyed this specific interaction, as the radiolabeled UL42 polypeptide was not significantly immunoprecipitated in the presence of Pol (Fig. 3bB and C). Indeed, larger quantities could be precipitated in the presence of β -galactosidase. A UL42 mutant truncated at both the N and C termini (Δ N35 Δ C282) was weakly coprecipitated by the anti-Pol serum, indicating that it retained some Pol-binding ability (Fig. 3bD).

The results of these experiments suggested that the Pol-binding site of UL42 was situated between residues 10 and 340, with some activity residing upstream of amino acid 269. Therefore, we next tested the effects of the set of in-frame internal deletions. Figure 4 shows the results obtained with five of these mutants. In general, most internal deletions of UL42 resulted in the loss of Pol-binding ability, as shown by the lack of significant coprecipitation of mutants Δ 20-131 (Fig. 4A), Δ 161-223 (Fig. 4B), Δ 250-308 (Fig. 4D), and Δ 308-356 (Fig. 4E) by the anti-Pol- β -galactosidase sera. A variety of other internal deletions summarized in Fig. 1 were assayed for heterodimerization but also resulted in polypeptides unable to bind Pol. However, the two smallest deletions tested, Δ 9-20 (Fig. 1) and Δ 242-250 (Fig. 4C), retained the ability to form a stable complex with Pol, much as they retained the ability to bind DNA.

We also examined the effects of 17 separate four-amino-acid insertions on heterodimerization. Sixteen of the linker insertions bound Pol with wild-type efficiency (Fig. 4F [I-131] and H [I-250]; Table 1). However, the insertion mutant I-160 was not precipitated by the anti-Pol sera in the presence of Pol, indicating that it could no longer form a stable complex with Pol (Fig. 4G). The results of these experiments are summarized in Fig. 1 and Table 1.

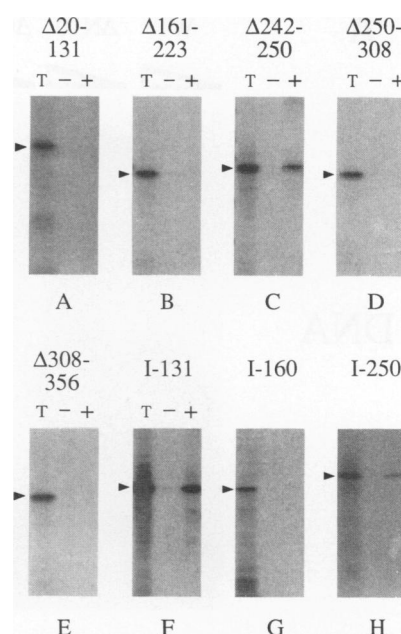


FIG. 4. Ability of UL42 mutants containing in-frame internal deletions or linker insertion mutations to bind Pol. The indicated UL42 mutants were tested for their Pol-binding activity as described in the legend to Fig. 3. The samples in panel H were electrophoresed on a higher-percentage gel than were the others.

Ability of selected UL42 mutants to increase the length of DNA product synthesized by Pol. UL42 has been shown to enhance synthesis of longer DNA products from a uniquely primed ssDNA template by Pol through an increase in processivity (20). Therefore, we tested the ability of our panel of UL42 mutants to increase the size of DNA product synthesized by Pol from a singly primed M13 ssDNA template. Aliquots of each mutant synthesized in rabbit reticulocyte lysate were incubated with 50 fmol of the primed template and 25 fmol of Pol purified from a baculovirus overexpression system in the presence of all four deoxynucleoside triphosphates (dNTPs), including [α - 32 P]dCTP. Newly synthesized ssDNA was then detected by autoradiography after fractionation on an alkaline agarose gel. This approach is similar to that of Gallo and coworkers (16), in which cell-free translated UL42 was used to stimulate Pol activity on an activated DNA template. However, our assay uses purified Pol and a template that more directly reflects the processivity of DNA synthesis. Figure 5 shows the results obtained with wild-type and five mutant UL42 polypeptides. Purified Pol synthesized only short lengths of DNA when incubated with water-programmed reticulocyte lysate (Fig. 5A, lanes n, o, and v; the small amount of material migrating slightly faster than full-length M13 in lane v results from incomplete denaturation of elongated primer-template duplex), whereas reticulocyte lysate alone had no detectable Pol activity (lane m). However, as increasing amounts of wild-type UL42-programmed reticulocyte lysate was added to purified Pol, synthesis of small DNA products was reduced and replaced by production of substantially longer strands, including some full-length M13 (lanes a to c). Therefore, in vitro-expressed UL42 appears to increase the processivity of purified Pol. Similarly, deletion mutants Δ N10 (lanes d to f) and Δ C340 (lanes j to l) and insertion mutant I-131 (lanes p to r) also increased Pol-mediated

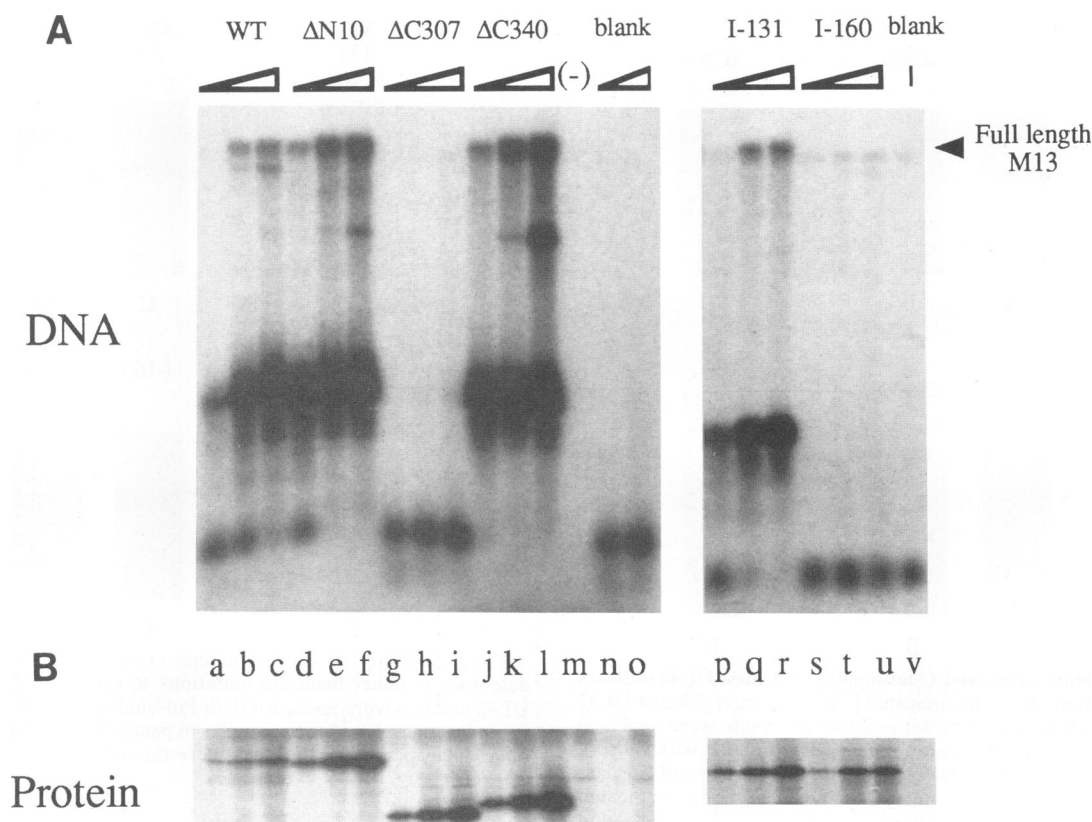


FIG. 5. Ability of UL42 mutants to increase the length of the DNA strand synthesized by Pol. Aliquots of rabbit reticulocyte lysate programmed with water (blank) or with mRNA encoding the indicated UL42 polypeptides were incubated with 25 fmol of purified Pol [except for lane m (–), in which Pol was omitted], 50 fmol of a singly primed M13 ssDNA template, and all four dNTPs, including [α - 32 P]dCTP, as described in Materials and Methods; 1-, 2.5-, and 5- μ l aliquots of lysates expressing UL42 polypeptides were used; while the samples run in lanes m, o, and v contained 5 μ l of blank lysate, and the sample in lane n contained 1 μ l. (A) Samples were analyzed for their content of [α - 32 P]dCTP-labeled DNA by electrophoresis on a 1.3% alkaline agarose gel after proteinase K digestion. Migration of full-length replicated M13 is indicated by the arrowhead. (B) Samples were analyzed for their content of [35 S]methionine-labeled protein by autoradiography after SDS-PAGE. WT, wild type.

synthesis of longer DNA strands while decreasing the amount of short products. In contrast, addition of deletion mutant Δ C307 (lanes g to i) or insertion mutant I-160 (lanes s to u) did not increase the length of DNA synthesized by Pol. To rule out the possibility that this defect resulted from instability or underexpression of the polypeptides, we analyzed aliquots of the Pol assays after the reaction period by SDS-PAGE for the presence of the [35 S]methionine-labeled UL42 mutants. As shown in Fig. 5B, the Δ C307 and I-160 polypeptides were present in amounts similar to those of mutants which were able to mediate synthesis of longer DNA products.

We also tested the other UL42 mutants for the ability to increase the length of DNA polymerization product synthesized by Pol. We found that the 13 insertion and 5 deletion mutants that were unaffected for both Pol and DNA binding also functioned normally to increase the length of DNA product synthesized by Pol (Fig. 1; Table 1). Interestingly, insertion mutants I-17 and I-20 and the deletion mutants Δ N10 and Δ 9–20, which showed an altered interaction with DNA, also behaved like wild-type UL42 (Fig. 5, lanes d to f; Fig. 1; Table 1). The 14 deletion mutants that bound Pol and DNA weakly or negligibly did not affect the polymerization activity of Pol (Fig. 1). These results are consistent with but substantially extend those of Gallo and coworkers (16), who

found that a UL42 mutant lacking 28 residues from the C terminus could still stimulate Pol activity, while one truncated at amino acid 249 and one equivalent to our Δ 35–280 could not.

Transient complementation of a UL42 null mutant. To assess the effect of the UL42 mutations on virus replication in vivo, we tested the ability of selected plasmids containing mutant *UL42* genes to complement the replication of virus Cgal Δ 42. Cgal Δ 42 contains a large deletion within the UL42 coding region and therefore cannot replicate in the absence of an exogenously supplied source of UL42 (23). Accordingly, Vero cells were transfected with the UL42-encoding plasmids and then superinfected with Cgal Δ 42. Any resulting virus progeny were then harvested and titered on the cell line V9, which contains a resident copy of the *UL42* gene and is therefore permissive for the growth of Cgal Δ 42. Table 2 shows the results obtained with a subset of the UL42 mutants. Insertion mutant I-131 and the two carboxy-terminal deletion mutants Δ C441 and Δ C340, which all bound Pol and DNA normally and increased the length of DNA product polymerized by Pol, substantially complemented the growth of Cgal Δ 42. However, mutant I-160, which was able to bind DNA but unable to bind Pol or to function as a processivity factor, failed to complement replication of the *UL42* null mutant. Deletion mutant Δ C307, which bound Pol weakly

TABLE 2. Ability of selected UL42 mutants to complement a UL42 null mutant

Gene	% Complementa ^a
ΔC441	51
ΔC340	35
ΔC307	0.04
ΔN160	0.2
Δ114-159	0.02
I-131	61
I-160	0.2
No plasmid	0.0013

^a Percentage relative to the complementation seen with the wild-type gene. All results are averages of at least two independent experiments. Figures in italics are considered to represent substantially lower than wild-type complementation.

but was unable to bind DNA or to function as a processivity factor, also failed to complement replication of the null mutant, as did the mutants ΔN160 and Δ114-159, which were impaired in all three in vitro assays.

DISCUSSION

The DNA-binding domain of UL42. In the assay for DNA binding used in this study, wild-type UL42 synthesized in rabbit reticulocyte lysate bound to dsDNA-cellulose in 50 mM NaCl and was eluted predominantly by 250 mM NaCl. Other studies have reported elution of UL42 from DNA-cellulose columns only at higher concentrations of NaCl (32). However, this discrepancy most likely reflects the stepped elution gradient used here, as UL42 purified from a baculovirus overexpression vector eluted similarly to the cell-free translated UL42 in this assay but at higher salt concentrations when a continuous gradient was used (45).

C-terminal truncation of UL42 indicated that the C-terminal one-third of the polypeptide was not necessary for dsDNA binding, as mutant ΔC340 eluted from a dsDNA-cellulose column indistinguishably from wild-type UL42. Further truncation of the C terminus, as in mutant ΔC307, destroyed the ability of the polypeptide to bind DNA, suggesting that residues between amino acids 308 and 340 play at least an indirect role in nucleic acid contact. In contrast to the relative insensitivity of the C terminus to truncation, the N-terminal insertion mutations I-17 and I-20 and the deletion mutations ΔN10 and Δ9-20 all affected DNA binding. All four mutants showed a biphasic fractionation profile from the dsDNA-cellulose column, with one population of material eluting normally at 250 mM and one eluting at 1 M NaCl. This finding suggests that this region of the polypeptide affects DNA-binding properties. However, mutant ΔN281 was also retained by the dsDNA-cellulose column and eluted predominantly at 500 mM NaCl. This finding is consistent with the importance of residues upstream of amino acid 340 inferred from the analysis of C-terminally truncated polypeptides but surprising in view of the cluster of N-terminal mutations that affected interaction with DNA, and also because the larger C-terminal fragments ΔN132 and ΔN160 and several internal deletion mutants retaining residues 281 to 448 lacked significant DNA-binding ability. The behavior of the latter mutants could result from misfolding of the polypeptide induced by the extra upstream sequences. The central regions of UL42 also appeared important for DNA binding, as all internal deletions tested except the small deletion Δ242-250 failed to bind DNA.

However, since these mutants also failed to bind Pol, as suggested above, they may be misfolded polypeptides and therefore provide little information on the direct function of the deleted residues.

One possible interpretation of the results presented above is that UL42 contains two regions that influence DNA binding: one site downstream of amino acid 281 that in some sequence contexts is sufficient to bind DNA alone, and a site closer to the N terminus that includes but is not limited to the first 20 residues that may contact DNA as well or otherwise modulate the activity of the C-terminal region indirectly. In this respect, it is interesting that ΔN281 eluted from DNA at a higher NaCl concentration than did full-length UL42, while several N-terminal mutations also showed a partial increase in salt resistance. This interpretation requires that in the cases in which sequences outside of amino acids 281 to 340 were deleted with loss of DNA-binding function, the mutant polypeptide was incorrectly folded. The fact that in every such instance Pol binding was also lost is consistent with this proposal, but further analysis is necessary to test the validity of this hypothesis. It is also interesting to note that the N-terminal one-quarter of UL42 contains a region of weak sequence similarity with the DPS polypeptide, a dsDNA-binding protein from *E. coli* (1).

The Pol-binding domain of UL42. The analysis of UL42 deletion mutants suggests that the Pol-binding site is contained between residues 21 and 340, since separate removal of the first 20 and last 148 residues of the polypeptide did not affect complex formation. Additional removal of C-terminal sequences in mutants ΔC307, ΔN35ΔC282, and ΔC269 reduced heterodimer formation but did not totally destroy it, implying that at least part of the Pol-binding site lies upstream of amino acid 269. Interestingly, these three mutants had little or no detectable DNA-binding activity, suggesting that the two activities are separable, although it is difficult to gauge the sensitivities of the assays used. Consistent with an N-terminal Pol-binding domain, most internal deletions within the N-terminal 350 residues prevented heterodimerization, and the only 1 of 17 four-amino-acid-insertion mutations to affect Pol association was at position 160. I-160 bound DNA normally, indicating that its inability to form a stable complex with Pol did not result from global misfolding. This result also confirms that the activities of DNA and Pol binding are separable. It is premature to conclude that the I-160 mutation lies directly within the protein-protein contact site, but as shown in Fig. 6, I-160 does fall within a highly hydrophilic stretch of amino acids and may therefore be a plausible candidate for a solvent-exposed binding site.

The UL42-binding site on Pol has been characterized in some detail. Previous work showed that the C-terminal 227 amino acids of Pol are sufficient to bind UL42 (14), and a more recent study has demonstrated that the final 35 amino acids of Pol are crucial for complex formation (13). This region of Pol contains a 3, -4 hydrophobic repeat and is predicted to be predominantly α helical by the Robson-Garnier secondary-structure prediction algorithm (13, 18). This finding raised the possibility that Pol and UL42 heterodimerize by a coiled-coil hydrophobic interaction, similar to that seen in many fibrous proteins and the leucine zipper class of transcriptional activators (10, 27, 37). The best candidate for a matching amphipathic α -helical region in UL42 lies between residues 242 and 253 (there are other regions of UL42 that contain a 3, -4 repeat of hydrophobic amino acids but which are not predicted to be α helical by Robson-Garnier analysis). However, as shown in Fig. 3, the major portion of this area was deleted in the mutant Δ242-

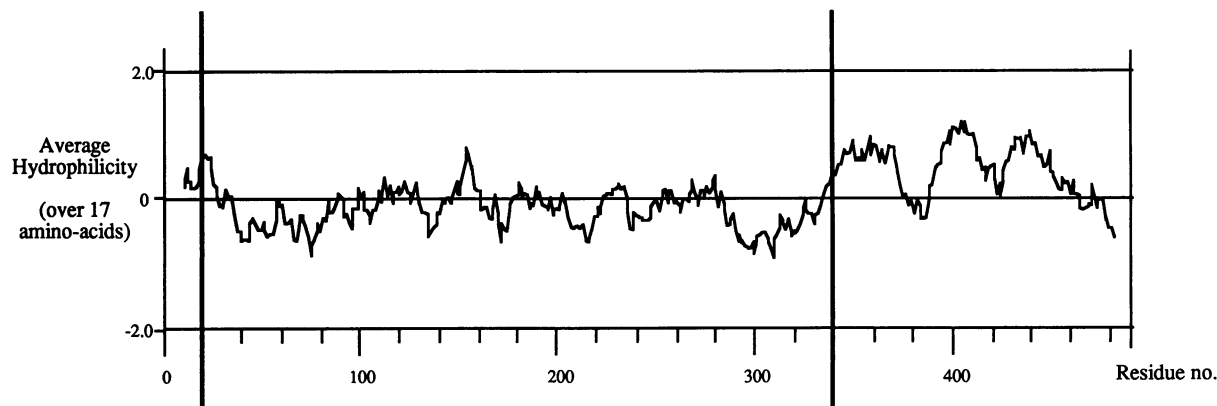


FIG. 6. Hydrophilicity plot of UL42. The predicted sequence of HSV strain 17 UL42 was analyzed for hydrophilicity, using the Hopp-Woods algorithm and a window size of 17 residues. Thick vertical bars indicate the extent of N- and C-terminal truncations that were tolerated without loss of protein activity.

250 without affecting Pol binding. Therefore, we do not currently favor the coiled-coil model of interaction. Interestingly, the hydrophilic region around amino acid 160 (residues 148 to 163) is strongly predicted to be α helical, raising the possibility of a more novel polar or charged interaction between two helices.

Possible structure of UL42. In the absence of truly accurate methods of predicting protein conformation, some predictions can nevertheless be made from cautious sequence analysis interpreted in the light of experimental data. For example, hydrophilic stretches of residues may function as linker regions between independently folded domains of a polypeptide or may be surface exposed loops on one domain (41). The results of genetic, biophysical, and biochemical experiments can be used to decide between these alternatives. Figure 6 shows a hydrophilicity plot for strain 17 UL42 (33) generated by using the Hopp-Woods algorithm (22), which predicts that the polypeptide has hydrophilic stretches at both the N and C termini and, internally, centered around amino acid 155. The vertical bars indicate the extent of N- and C-terminal deletions that were tolerated without loss of DNA- or Pol-binding function and also show a close correlation with the hydrophilic terminal regions.

The hydrophilic region around amino acid 155 could potentially indicate a boundary between N-terminal and central domains of the protein. However, we prefer to interpret it as a surface-exposed region on a single domain, since a variety of deletion mutants lacking sequences either N or C terminal to amino acid 155 were globally defective in the assays used in this study. The fact that the only linker insertion mutation to affect Pol binding (but not DNA binding) lay within this hydrophilic stretch also supports this hypothesis. Therefore, we propose that UL42 consists of an N-terminal domain composed approximately of the first 308 to 340 amino acids and a C-terminal region including the final 148 residues. The N-terminal domain includes both Pol- and DNA-binding functions, is sufficient to increase the apparent processivity of the Pol subunit, and can also complement replication of a UL42 null mutant. This region of UL42 is also the most highly conserved in open reading frame (ORF) 16, encoding the corresponding varicella-zoster virus protein (12). The C terminus of UL42 is not required for any of the assayed functions in vitro and also seems to be nonessential for replication in tissue culture. It is also highly hydrophilic (Fig. 6) and rich in proline residues, so it may not fold

compactly in the true sense of a domain. The results of partial proteolytic digestion experiments are consistent with the predictions presented above, since the major stable proteolytic fragments generated by a variety of proteases arose from the N-terminal two-thirds of UL42, while no stable fragments including the C terminus of the protein were observed (26). Although the proposed N-terminal domain envelops Pol- and DNA-binding activities, the two functions are clearly separable, since an insertion mutation at position 160 destroyed heterodimerization without affecting DNA binding. Similarly, several mutations at the N terminus altered DNA binding but not complex formation, and progressive truncation of the C terminus abrogated DNA binding before Pol binding in our assays.

Mechanism of UL42 as a processivity factor. Previous studies have shown that Pol synthesizes longer DNA products in the presence of UL42 (20, 21) and that this activity results from an increase in processivity (20). Therefore, it seemed likely that this activity resulted from a specific interaction between the two polypeptides, although some workers have reported stimulation of HSV Pol by unrelated DNA-binding proteins (35). The failure of mutant I-160 to increase the size of DNA products synthesized by Pol argues that the UL42-mediated increase in processivity directly requires the Pol-UL42 interaction, since I-160 bound DNA normally but did not bind Pol. In addition, the failure of I-160 to complement growth of a virus lacking a functional copy of the UL42 gene confirms that the Pol-UL42 interaction is necessary for virus replication. This result is consistent with the observed phenotypes of Pol mutants unable to bind UL42 (13) and therefore validates the Pol-UL42 interaction as a target for directed drug design (14).

We generated three mutants that retained a low level of Pol-binding activity but had minimal DNA-binding ability (Δ C307, Δ N35 Δ C282, and Δ C269). None of these mutants were able to increase the apparent processivity of Pol, but they do not conclusively test the hypothesis that UL42 increases the processivity of Pol by tethering it to the DNA template (20), as their marked reduction in Pol binding could also have caused the defect. However, all of the mutants which associated with both Pol and DNA also increased the length of DNA synthesized by Pol, which is consistent with but does not prove the idea that Pol binding and DNA binding are sufficient for function as a processivity factor.

If UL42 does increase the processivity of Pol through its

own DNA-binding activity, then it is interesting to note that this implies a different mode of action to the best-characterized Pol processivity factor, the β subunit of *E. coli* Pol III (Pol III- β). Pol III- β has no intrinsic affinity for DNA (42) but rather dimerizes to form a torus that is sterically retained on the nucleic acid strand (24). The DNA-binding model for UL42 as a processivity factor also raises the problem of how the protein slides to follow the elongating primer without acting as a brake on Pol.

Other known processivity factors include the gene 45 protein of bacteriophage T4 and the proliferating cell nuclear antigen of eukaryotic Pol δ , but similarly to Pol III- β , gene 45 and proliferating cell nuclear antigen have little affinity for DNA (3, 25, 28, 34); in fact, an analogous structure has been proposed for the three polypeptides (24). However, as eukaryotic Pol δ is also a heterodimer of ≈ 125 - and 50-kDa subunits (4), possibly a better cellular analog or homolog for UL42 may be the as yet uncharacterized 50-kDa subunit.

Does UL42 play a role other than as a processivity factor? In the course of a normal productive HSV infection, Pol expression peaks early in infection and is then turned off at late times via translational regulation (46, 47). However, synthesis and accumulation of UL42 continues at late times, leading to an excess of UL42 over that needed for a 1:1 complex with Pol (19, 36). This raises the possibility that the free UL42 performs an additional function to the heterodimerized form, though it should be noted that a cell line expressing approximately 1% of the normal levels of UL42 efficiently complements the growth of a UL42 null virus (23). The C-terminal 150 amino acids of UL42 are apparently not involved in increasing the processivity of Pol, so it is tempting to speculate that this region of the protein may be involved in such alternative functions. In support of this idea, many of the counterparts of UL42 in other herpesviruses such as varicella-zoster virus (ORF 16), Epstein-Barr virus (BMRF1), and cytomegalovirus (UL44) contain a hydrophilic region at the C terminus that is similarly proline rich in ORF 16 and BMRF1 and glycine-rich in UL44 (2, 7, 12, 15). Such conservation of structure is suggestive of a functional role for the C-terminal domain. However, this region of UL42 is clearly not essential for virus replication in Vero cells, as mutant Δ C340 complemented growth of a UL42 null virus, albeit at less than wild-type efficiency (Table 2). Therefore, if the C terminus of UL42 does have a function, it may be subtle or required in cells other than Vero cells. Construction of a recombinant virus containing the appropriately truncated UL42 gene should resolve this question.

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